

EXHIBIT 41

Nucleic Acid Probes

Editor

Robert H. Symons, Ph.D.

Professor of Biochemistry
University of Adelaide
Adelaide, S. A., Australia



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virus or viroid concentration in sap is relatively high, but it is where the concentration is low and it is important to detect all infected plants. Examples of the latter are the detection of ASBV in avocado trees^{5,41} and of BYDV in cereals.^{22,23} In these cases, a deproteinization step and concentration of the nucleic acids prior to dot-blot assay are needed.

There is certainly a need for simpler extraction procedures, preferably with as few steps as possible and avoidance of the use of phenol for deproteinization. For large numbers of samples, the tissue extraction must be simple since usual laboratory procedures such as homogenization in a blender or grinding in a mortar and pestle are impractical. In our hands, the roller sap extractor (Erich Pollahne, F.R.G.) is very efficient and can be used for soft tissues such as cereals²³ and leaves of wooly trees, such as avocado and oil palm.¹⁰¹

2. *Nonradioactive Probes*

The need for future routine diagnosis to be based on nonradioactive procedures is obvious. It is desirable to develop probe technology where the signal is not an insoluble colored product on a membrane filter but a soluble colored product or some other soluble product, both of which can be measured by suitable means and the result printed out. This approach is essential for the automation of the detection system.

3. *Alternatives to Dot-Blots*

Dot-blot assays are tedious in requiring the spotting of a small volume of each sample on to a membrane filter, baking to immobilize the nucleic acids, prehybridizing and then hybridizing before washing and color development. The use of plastic bags for the hybridization steps is another part of the procedure which needs eliminating.

a. *Two-Phase Sandwich Hybridization*

This method was developed by Ranki et al.¹⁰² for the detection and quantitation of nucleic acids in crude clinical samples using adenovirus DNA as a model.¹⁰³ As far as we are aware, it has not been used for the detection of plant virus nucleic acids. The general principle of the method is outlined in Figure 5A using nonradioactive single-stranded DNA probes in the phage M13 DNA vector as an example. Two probes are required which hybridize to different, nonoverlapping regions of the target nucleic acid. One probe is bound to a solid support such as nitrocellulose by standard procedures while the other is labeled with biotin using, for example, Photobiotin® (Chapter 2, Section III.A.2). The probe-bound filter is hybridized with the test nucleic acid sample in the presence of the second biotin-labeled probe which can only be bound to the filter via the bridge of target nucleic acid. After the usual washing procedures, the biotin-labeled probe is detected by standard procedures (Chapter 2, Section V.A). It should be appreciated that each sample must be hybridized separately. Thus, Ranki et al.¹⁰² used DNA bound to 10 mm diameter nitrocellulose discs and carried out the hybridization in 0.4 ml in small plastic ampoules at 65°C, usually overnight.

The method has been successfully used in the detection of adenovirus DNA in nasopharyngeal mucus aspirates using ¹²⁵I-DNA probes,¹⁰⁴ cytomegalovirus in urine,¹⁰⁵ and the *ompA* gene in *E. coli*.¹⁰⁶ Further examples are given in Chapter 3. In an extension of this approach, Polsky-Cynkin et al.¹⁰⁷ have investigated model sandwich hybridization using DNA probes bound to polypropylene tubes and receptacles and to CNBr-activated Sepharose-4B beads with encouraging results. It appears feasible that this sandwich hybridization technique may have a role in the routine diagnosis of plant viral nucleic acids, especially if it can be readily adapted to nonradioactive detection methods.

b. *Single-Phase Sandwich Hybridization*

A viable long term alternative is for both the target nucleic acid and the two probes to hybridize *in solution*. Such a system would offer much faster hybridization because the rate

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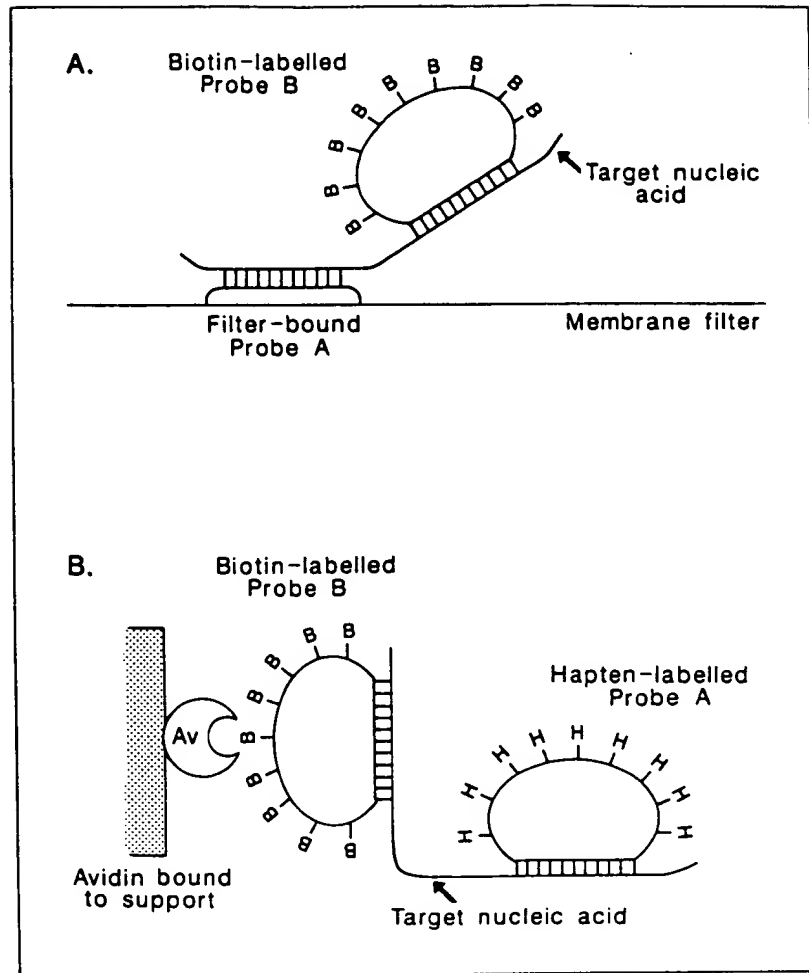


FIGURE 5. Schematic diagrams showing the general principles of two alternative methods of sandwich hybridization: (A) two-phase sandwich hybridization; (B) single-phase sandwich hybridization. The two probes shown here, as examples, are single-stranded DNA probes in the phage M13 DNA vector, one labeled with biotin (B), and the other with a hapten (H). See text for further details.

of hybridization in a single-phase system is a lot greater than in the usual two-phase dot-blot hybridization.¹⁰⁸⁻¹¹¹ In addition, there is the potential for greater flexibility,^{103,112} e.g., the size of the sample may be increased with consequent increase in final detection signal.

The critical step in this method is to conveniently and efficiently separate the probe: target hybrids from the unhybridized probe prior to signal detection. This can be achieved by adsorption of the hybrid complex to a solid support, followed by thorough washing. In the example in Figure 5B, support-bound avidin is used to adsorb the biotin-labeled probe B in the hybrid complex, an extraction process which we find may only be about 30% efficient.¹¹² Detection is via the hapten-labeled probe A and, for example, an enzyme coupled antibody.

The system described has considerable potential for the routine diagnosis of plant viruses using nonradioactive probes. Although the hybridization reaction is straight forward, the extraction and detection procedures are where rapid and reliable developments need to be made.